DESCRIPTION

MATERIALS AND METHODS FOR PROVIDING PLANTS WITH INCREASED RESISTANCE TO ENVIRONMENTAL STRESS

Cross-Reference to a Related Application

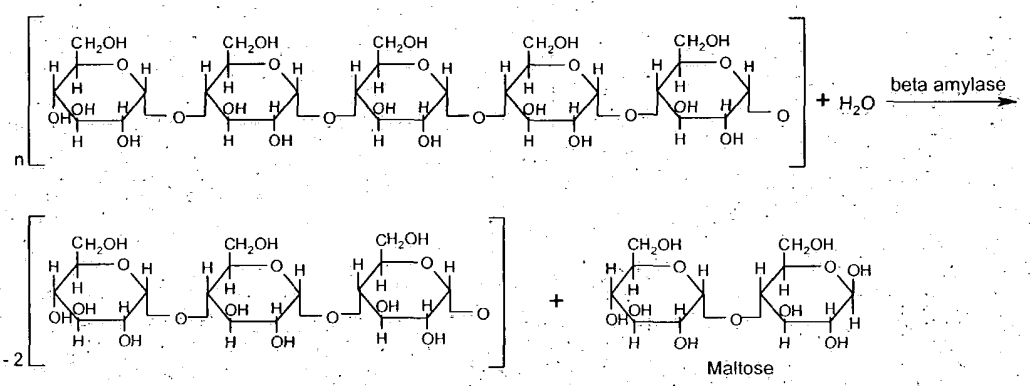
This application claims the benefit of U.S. Provisional Application Serial No. 60/390,384, filed June 21, 2002.

Background of Invention

A variety of factors are involved in starch degradation in plants. Diastase is a group of enzymes that catalyze the breakdown of carbohydrates. Diastase enzymes include alpha-amylase, beta-amylase, starch phosphorylase, and starch debranching enzyme (DBE) or R-enzyme. Maltose is either a major product or initial product of starch degradation by such enzymes.

Alpha-amylase (α-amylase) hydrolyzes starch, glycogen, and related polysaccharides by randomly cleaving internal α-1,4 glucosidic linkages. It is widely distributed in plants and has a major role in the utilization of polysaccharides. Under anoxic conditions, rice seeds are able to induce α-amylase.

Beta-amylase (β-amylase) is an exoamylase that hydrolyses α-1,4 glycosidic linkages of polyglucan chains at the non-reducing end to produce maltose (4-O-α-D-Glucopyranosyl-β-D-glucose) (see Scheme 1 below). The primary physiological role of β-amylase is considered to be in starch breakdown (Beck and Ziegler 1989).



Scheme 1. β-amylase hydrolysis of α-1,4 glycosidic linkage of a polyglucan chain.

The complete physiological role of β-amylase in starch breakdown remains unclear because this enzyme is considered by some, based on *in vitro* studies, to be unable to attack native starch without prior digestion by other amylolytic enzymes like α-amylase (Beck and Ziegler, 1989). Current models of β-amylase function favor its action to be predominantly on polyglucan chains. β-amylase randomly encounters polyglucan chains and hydrolyzes sequentially after complexing with the polymeric glucan chains (Adachi *et al.*, 1998).

The enzyme β-amylase and its activity is found in seeds, tubers, and leaves; everywhere starch is present in plants (Avigad and Dey 1997). In leaves, this enzyme has been localized to phloem sieve tubes (Wang *et al.*, 1995), the cytoplasm of mesophyll cells (Monroe and Preiss, 1990), and in the stroma of chloroplasts (Scheidig *et al.*, 2002; Lao *et al.*, 1999). One Arabidopsis β-amylase, designated ct-Bmy (AJ250341; BMY8), has been biochemically localized to the chloroplast stroma (Lao *et al.* 1999) in import studies with isolated pea chloroplasts, and confirmed by accumulation of a β-amylase-GFP fusion protein in Arabidopsis chloroplasts (Lao *et al.* 1999). Database searches have revealed that Arabidopsis contains nine β-amylases, designated BMY1 to BMY9. BMY1, which is also called ram1, accounts for more than 80 % of total β-amylase activity in Arabidopsis and is localized to the vacuole or the secretory pathway in mesophyll cells. BMY2 to BMY6 are localized to the cytosol, based on sequence analysis with Target P. BMY7, BMY8, and BMY9 contain a putative chloroplast targeting peptide.

Beta-amylase expression is induced under stress conditions such as drought (Todaka *et al.*, 2000), cold (Nielsen *et al.*, 1997), and heat stress (Dreier *et al.*, 1995). When Arabidopsis plants are cold stressed at 4°C for 12 hours, β-amylase (AJ25034; ct-Bmy or BMY8) expression increases about 14-fold (Sung 2001) and induction can occur as early as 2 hr after exposure to cold stress (Seki *et al.* 2001). During water stress, β-amylase activity increases and is followed by an increase in the free sugars, sucrose and maltose in cucumber cotyledons (Todaka *et al.*, 2000). Similarly, when pearl millet, maize (Datta *et al.*, 1999) and barley (Dreier *et al.*, 1995) are exposed to water and salt stress, β-amylase activity is similarly induced. The increase in the β-amylase activity is correlated with increased β-amylase protein. When potato tuber storage temperature is reduced from 20°C to 5°C or 3°C, β-amylase activity is increased four to five fold within 10 days, whereas the activity of α-glycosidase, which hydrolyzes α 1,1; α 1,2; α 1,3; α 1,4, and α 1,6 glycosidic bonds, and endoamylase, which hydrolyzes internal α 1,4 glycosidic linkages of starch, is not changed. However, the role of β-amylase under temperature shock is unknown. Chloroplast localized β-amylase has been shown to be strongly stress induced in a number of cold and heat stress related microarray studies (Seki *et al.*, 2001; Sung 2001; Seki *et al.*, 2002; Fowler and Thomashow 2002; Kreps *et al.*, 2002).

Additional enzymes that produce maltose during the breakdown of starch include starch phosphorylase and starch DBE. Starch phosphorylase cleaves and phosphorylates terminal glucose residues in starch, only cleaving α-1,4 linked residues and not α-1,6 or α-1,4 close to α-1,6 linkages. Starch DBE hydrolyzes α-1,6 linkages in glucans. In particular, DBE is active on amylopectin, α-limit dextrins, and phosphorylase-limit dextrins.

Compatible solutes (osmoprotectants, osmolytes) are low molecular weight organic molecules that accumulate under stress conditions. Examples of compatible solutes include trehalose, sucrose, glucose, and fructose. They help to stabilize proteins and membranes and contribute to cell osmotic pressure under stress conditions (Yancey *et al.*, 1982). There are three general types of osmoprotectants: methylamines (betaines), amino acids (proline), and polyols (glycerol, sucrose) (Yancey *et al.*, 1982).

In many plants, sucrose is a compatible solute that is considered to stabilize membranes and proteins during temperature stress and water stress (Santarius, 1973). Sucrose accumulates in a wide range of plants during temperature stress (Wanner and Juntilla, 1999; Krapp and Stitt,

1995; Strand *et al.*, 1999; Strand *et al.*, 1997; Guy *et al.*, 1992). The activity and transcripts of sucrose biosynthetic enzyme, sucrose phosphate synthase (SPS), are known to increase under temperature stress (Guy *et al.*, 1992; Strand *et al.*, 1997). When Arabidopsis plants are exposed to low temperature (5°C), within a few hours sugar accumulation follows consisting primarily of sucrose, glucose, and fructose (Guy *et al.*, 1992; Wanner and Juntilla, 1999), phosphorylated hexose intermediates (Strand *et al.*, 1997; Strand *et al.*, 1999; Krapp and Stitt, 1995). The increase in sucrose is well correlated with an increased expression (Strand *et al.*, 1997) and activity of SPS (Guy *et al.*, 1992).

Proline is another compatible solute that accumulates under stress conditions such as salt, dehydration, and cold stress (Verbruggen *et al.*, 1993; Yoshiba *et al.*, 1995; Xin and Browse, 1998; Wanner and Juntilla, 1999; Gilmour *et al.*, 2000). Δ^1 pyrroline-5-carboxylate synthetase (P5CS) catalyzes the first two steps in proline biosynthesis in plants (Hu *et al.*, 1992). It is suggested that P5CS plays a key role in proline biosynthesis under osmotic stress, and catalyzes the major regulated step (Yoshiba *et al.*, 1995; Strizhov *et al.*, 1997).

Acute temperature shock is likely to induce deleterious effects in all parts of a cell. Since compatible solutes contribute to acquired thermotolerance (Singer and Lindquist, 1998), as well as acquired freezing tolerance mechanisms (Guy, 1990), then accumulation of one or more compatible solutes in the chloroplast plays a critical part of a multi-faceted network of molecules, stress proteins, biochemical transformations and physiological adjustments that collaborate to preserve the viability of an organelle, a cell, a tissue or an organism during transient periods of stress that would be otherwise lethal (Thomashow, 1999).

Brief Summary of the Invention

The subject invention pertains to materials and methods for providing plants, plant tissue, plant cells, and plant organelles, such as chloroplasts, with increased resistance to thermal (heat and/or cold) stress, and other forms of environmental stress such as water, salt stress, nutritional stress, aerobic and anaerobic stress, and wounding. In one embodiment, a plant is transformed with a polynucleotide that encodes a protein that produces, or catalyzes the synthesis of, or results in the production of maltose or a maltose alcohol. In an exemplified embodiment, the polynucleotide encodes a diastase enzyme, or an enzymatically active fragment thereof.

The subject invention also concerns plants, plant tissue, and plant cells transformed with or bred to contain a polynucleotide that encodes a protein that produces, or catalyzes the synthesis of, or results in the production of maltose or a maltose alcohol. In an exemplified embodiment, the polynucleotide encodes a β-amylase enzyme. In one embodiment, the polynucleotide is overexpressed in the plant in order to elevate the level of maltose or maltose alcohol in a plant.

Brief Description of the Sequences

SEQ ID NO. 1 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 2 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 3 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 4 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 5 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 6 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 7 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 8 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 9 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 10 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 11 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 12 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 13 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 14 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 15 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 16 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 17 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 18 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 19 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 20 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 21 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 22 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 23 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 24 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 25 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 26 is an oligonucleotide used in PCR amplification.

SEQ ID NO. 27 is a transit peptide encoding sequence that encodes the amino acid sequence shown in SEQ ID NO. 28 that can be used according to the present invention.

SEQ ID NO. 28 is a transit peptide sequence that can be used according to the present invention.

SEQ ID NO. 29 is a transit peptide encoding sequence that encodes the amino acid sequence shown in SEQ ID NO. 30 that can be used according to the present invention.

SEQ ID NO. 30 is a transit peptide sequence that can be used according to the present invention.

SEQ ID NO. 31 is a transit peptide encoding sequence that encodes the amino acid sequence shown in SEQ ID NO. 32 that can be used according to the present invention.

SEQ ID NO. 32 is a transit peptide sequence that can be used according to the present invention.

Brief Description of Drawings

Figure 1 shows RT-PCR analysis of selected genes following heat and cold shock. Arrow head shows 18S rRNA internal control. BMY1, beta-amylase 1 (At4g15210); BMY7, beta-amylase 7 (At3g23920); BMY8, beta-amylase 8 (At4g17090); BMY9, beta-amylase 9 (At4g00490); AMY, alpha-amylase (At1g69830); IMY, isoamylase (At2g39930); Phos b, phosphorylase b (At3g29320); P5CS, delta-1-pyrroline 5-carboxylase synthetase (At2g39795); SPS, sucrose-phosphate synthase (AL391222); Hsp70, heat shock protein 70 (At3g12580); Cor78, low-temperature-induced protein 78 (At5g52310); 18S rRNA (At3g41768).

Figure 2 shows heat shock and cold shock time course experiments. RT-PCR analysis of selected beta-amylase genes was performed under conditions of heat shock and cold shock. Arrowhead shows 18S rRNA internal control.

Figures 3A-3B show time course experiments for soluble sugars and starch accumulation under conditions of heat shock and cold shock.

Figures 4A-4C show *in vitro* compatible solute assay for three enzymes. Figure 4A: SspI. Figure 4B: G6PDH. Figure 4C: ADH.

Figure 5 shows electron transport chain activity in the absence and presence of soluble sugars during heat shock.

Figure 6 shows electron transport chain activity in the absence and presence of soluble sugars following freezing stress.

Figures 7A-7C show time course compatible solute assay (A) SspI, arrowhead shows the 4 and 5 kb bands, (B) G6PDH, (C) ADH.

Detailed Disclosure of the Invention

The subject invention pertains to materials and methods for protecting plants and plant organelles, such as chloroplasts, during thermal (heat and cold) stress, and other forms of environmental stress such as water, salt stress, nutritional stress, aerobic and anaerobic stress, and wounding. It has been discovered that maltose accumulates in the chloroplast in response to acute temperature shock and can act as chloroplast compatible solute for this vital plant cell organelle. In one embodiment of the invention, a plant, plant tissue or plant cell is transformed with a polynucleotide that encodes a protein that produces, or catalyzes the synthesis of, or results in the production of maltose or a maltose alcohol, such as, for example, maltitol. Polynucleotides can be introduced into plant cells using standard techniques known in the art. In one embodiment, the polynucleotide is overexpressed in the plant in order to elevate the level of maltose or maltose alcohol in a plant. In an exemplified embodiment, the polynucleotide encodes a β-amylase enzyme, or an enzymatically active fragment thereof.

In another embodiment of the present invention, a polynucleotide encodes an α-amylase. The polynucleotide can comprise any sequence that encodes a protein that has α-amylase activity or that otherwise results in the production of maltose. Polynucleotide sequences encoding α-amylase can be synthesized as described in U.S. Patent Nos. 5,460,952; 5,498,832; and 5,712,112.

In another embodiment of the present invention, a polynucleotide encodes a DBE or encodes a protein that has DBE activity or that results in the production of maltose.

Polynucleotide sequences encoding DBE are well known in the art (see, for example, U.S. Patent Nos. 5,912,413 and 6,469,230).

The protein encoded by a polynucleotide of the present invention can be targeted for chloroplast localization. For example, the targeting sequence of a plant β-amylase can be used. The use of a targeting sequence heterologous to the protein encoded by the polynucleotide is also encompassed within the scope of the invention. Numerous examples of other amino acid sequences that when added on or included in a protein result in the transport of the protein to chloroplasts within a cell are known in the art (see, for example, U.S. Patent No. 6,489,540). For example, a protein can be directed to a chloroplast by incorporating a chloroplast transit peptide sequence, such as those sequences from ADPGPP, 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSP synthase), and SS RUBISCO, at the N-terminus of the protein when the protein is synthesized. Examples of chloroplast transit sequences are shown in SEQ ID NOs. 27-32.

In those embodiments where a polynucleotide of the present invention encodes a β-amylase, the polynucleotide can comprise any sequence that encodes a protein that has β-amylase activity or that otherwise results in the production of maltose. Polynucleotide sequences encoding β-amylase are well known in the art. U.S. Patent Nos. 5,762,057, 5,688,684, 5,863,784, and 5,082,781 describe nucleotide sequences encoding a β-amylase enzyme. Also contemplated within the scope of the invention are polynucleotides that encode β-amylase enzymes that have been modified so as to exhibit reduced inhibition by maltose. Also contemplated within the scope of the invention are polynucleotides that encode β-amylase enzymes that exhibit increased thermostability (Yoshigi *et al.*, 1995; Mikami *et al.*, 1999). In another embodiment, the polynucleotide further comprises a sequence encoding an inducible maltase enzyme which when expressed rapidly converts maltose to glucose.

In one embodiment of the subject method, a plant, plant tissue, or plant cell is transformed with a polynucleotide of the present invention and a transformed plant comprising the polynucleotide is grown from the transformed cell, tissue or plant.

The subject invention also concerns polynucleotide expression constructs comprising a polynucleotide sequence of the present invention. Expression constructs of the invention generally include regulatory elements that are functional in the intended host cell in which the expression construct is to be expressed in. Thus, a person of ordinary skill in the art can select

regulatory elements for use in bacterial host cells, yeast host cells, plant host cells, insect host cells, mammalian host cells, and human host cells. Regulatory elements include, for example, promoters, transcription termination sequences, translation termination sequences, enhancers, and polyadenylation elements. As used herein, the term "expression construct" refers to a combination of nucleic acid sequences that provides for transcription of an operably linked nucleic acid sequence. As used herein, the term "operably linked" refers to a juxtaposition of the components described wherein the components are in a relationship that permits them to function in their intended manner. In general, operably linked components are in contiguous relation.

An expression construct of the invention can comprise a promoter sequence operably linked to the polynucleotide sequence of the invention. Promoters can be incorporated into a polynucleotide using standard techniques known in the art. Multiple copies of promoters or multiple promoters can be used in an expression construct of the invention. In one embodiment, a promoter can be positioned about the same distance from the transcription start site as it is from the transcription start site in its natural genetic environment. Some variation in this distance is permitted without substantial decrease in promoter activity. A transcription start site is typically included in the expression construct.

Overproduction in a plant cell of a protein, such as a β-amylase or an α-amylase, encoded by a polynucleotide of the invention can be accomplished using polynucleotide constructs that have constitutive or inducible promoters operably linked to, for example, polynucleotides encoding β-amylase that are to be transformed into plants. Accordingly, promoters that drive constitutive expression and promoters that are inducible are contemplated within the scope of the invention. Constitutive promoters that can be used with the present invention include, but are not limited to, cauliflower mosaic virus (CaMV), nopaline synthase, ubiquitin, and actin promoters.

Inducible promoters that can be used with the present invention include those that are regulated by heat shock, freezing, drought, and salinity. Heat shock promoters that can be used with the subject invention include, but are not limited to, those disclosed in U.S. Patent Nos. 5,447,858 and 6,268,548, as well as the promoter from the *Hsp70* gene (Horvath *et al.*, 1993; Gilmour *et al.*, 1998; Lee *et al.*, 1996; and Kim *et al.*, 2002), or the *Hsp101* or *Hsp17.6* promoter.

Promoters that are inducible by cold temperature (*i.e.*, *cor78*) are also contemplated for use with the subject invention. For example, the following cold-regulated elements/promoters can be used with the subject invention: (1) cis-acting cold-regulatory element present in all plant cold-regulated promoters as disclosed by Yamaguchi-Shinozaki *et al.* (1994); Baker *et al.* (1994); Jiang *et al.* (1996); (2) the COR15a promoter (Baker *et al.*, 1994); (3) the COR78/RD29A promoter (Horvath *et al.*, 1993; Yamaguchi-Shinozaki *et al.*, 1994); (4) the COR6.6 promoter (Wang *et al.*, 1995a); (5) the KIN1 promoter (Wang *et al.*, 1995a) genes of *Arabidopsis*; (6) the BN115 promoter gene of Brassica napus (White *et al.*, 1994); (7) the genes encoding the proteins involved in cold adaptation in *Arabidopsis thaliana* as disclosed in U.S. Patent Nos. 5,296,462 and 5,356,816; the COR15b promoter; and the galactinol synthase promoter.

Promoters regulated by drought conditions including, for example, *rd29* of *Arabidopsis* (Yamaguchi-Shinozaki *et al.*, 1993), FL6-55 and FL2-56 (Seki *et al.*, 2001, can also be used with the subject invention. Further, promoters regulated by a combination of stresses are contemplated for used with the subject invention. For example, genes regulated by freezing, drought, and high salinity including, for example, *rd29A, cor78, kin1, kin2, cor15a, rd17,* and *erd10* in transgenic plants (Yamaguchi-Shinozaki, 2001; Baker *et al.*, 1994; Wang *et al.*, 1995; Iwasaki *et al.*, 1997), can be used with the subject invention.

Organ-specific promoters are also contemplated and include E8 promoter from tomato. Plant viral promoters can also be used, such as for example, the CaMV 35S (including the enhanced CaMV 35S promoter (see, for example U.S. Patent No. 5,106,739)) or CaMV 19S promoter. Examples of other plant promoters that can be used include, but are not limited to, prolifera promoter, Ap3 promoter, T-DNA 1'- or 2'-promoter of *A. tumafaciens*, polygalacturonase promoter, chalcone synthase A (CHS-A) promoter from petunia, tobacco PR-1a promoter, alcA gene promoter, pin2 promoter (Xu *et al.*, 1993), maize WipI promoter, maize trpA gene promoter (U.S. Patent No. 5,625,136), maize CDPK gene promoter, and RUBISCO SSU promoter (U.S. Patent No. 5,034,322).

Expression constructs of the invention may optionally contain a transcription termination sequence, a translation termination sequence, a sequence encoding a signal peptide sequence, such as a sequence encoding a chloroplast transit peptide (for targeting a protein to a plant cell

chloroplast), and/or enhancer elements. Transcription termination regions can typically be obtained from the 3' untranslated region of a eukaryotic or viral gene sequence. Transcription termination sequences can be positioned downstream of a coding sequence to provide for efficient termination. Signal peptides are a group of short amino terminal sequences that encode information responsible for the relocation of an operably linked mature polypeptide to a wide range of post-translational cellular destinations, ranging from a specific organelle compartment to sites of protein action and the extracellular environment. Targeting gene products to an intended cellular and/or extracellular destination through the use of operably linked signal peptide sequence is contemplated for use with the polypeptides of the invention. Enhancers are cis-acting elements that increase activity of a promoter and can also be included in the expression construct. Enhancer elements are known in the art, and include, but are not limited to, the CaMV 35S enhancer element, maize shrunken-1 enhancer element, cytomegalovirus (CMV) early promoter enhancer element, and the SV40 enhancer element.

DNA sequences which direct polyadenylation of mRNA transcribed from the expression construct can also be included in the expression construct, and include, but are not limited to, an octopine synthase or nopaline synthase signal. The expression constructs of the invention can also include a polynucleotide sequence that directs transposition of other genes, *i.e.*, a transposon.

Expression constructs of the invention can also include one or more dominant selectable marker genes, including, for example, genes encoding antibiotic resistance and/or herbicide-resistance for selecting transformed cells. Antibiotic-resistance genes can provide for resistance to one or more of the following antibiotics: hygromycin, kanamycin, bleomycin, G418, streptomycin, paromomycin, neomycin, and spectinomycin. Kanamycin resistance can be provided by neomycin phosphotransferase (NPT II). Herbicide-resistance genes can provide for resistance to phosphinothricin acetyltransferase or glyphosate. Other markers used for cell transformation screening include genes encoding β-glucuronidase (GUS), β-galactosidase, luciferase, nopaline synthase, chloramphenicol acetyltransferase (CAT), green fluorescence protein (GFP), or enhanced GFP (Yang *et al.*, 1996).

The subject invention also concerns polynucleotide vectors comprising a polynucleotide sequence of the invention. Unique restriction enzyme sites can be included at the 5' and 3' ends

of an expression construct or polynucleotide of the invention to allow for insertion into a polynucleotide vector. As used herein, the term "vector" refers to any genetic element, including for example, plasmids, cosmids, chromosomes, phage, virus, and the like, which is capable of replication when associated with proper control elements and which can transfer polynucleotide sequences between cells. Vectors contain a nucleotide sequence that permits the vector to replicate in a selected host cell. A number of vectors are available for expression and/or cloning, and include, but are not limited to, pBR322, pUC series, M13 series, and pBLUESCRIPT vectors (Stratagene, La Jolla, CA).

Polynucleotides of the present invention can be composed of either RNA or DNA. Preferably, the polynucleotides are composed of DNA. The subject invention also encompasses those polynucleotides that are complementary in sequence to the polynucleotides disclosed herein.

Because of the degeneracy of the genetic code, a variety of different polynucleotide sequences can encode a protein that produces, or catalyzes the synthesis of, or results in the production of maltose or a maltose alcohol, such as a β-amylase as disclosed herein. In addition, it is well within the skill of a person trained in the art to create alternative polynucleotide sequences encoding the same, or essentially the same, polypeptides of the subject invention. These variant or alternative polynucleotide sequences are within the scope of the subject invention. As used herein, references to "essentially the same" sequence refers to sequences which encode amino acid substitutions, deletions, additions, or insertions which do not materially alter the functional activity of the polypeptide encoded by the polynucleotides of the present invention.

Substitution of amino acids other than those specifically exemplified or naturally present in a polypeptide encoded by a polynucleotide of the invention are also contemplated within the scope of the present invention. For example, non-natural amino acids can be substituted for the amino acids of the native protein, so long as the protein having the substituted amino acids retains substantially the same biological activity as the native protein in which amino acids have not been substituted. Examples of non-natural amino acids include, but are not limited to, ornithine, citrulline, hydroxyproline, homoserine, phenylglycine, taurine, iodotyrosine, 2,4-diaminobutyric acid, α-amino isobutyric acid, 4-aminobutyric acid, 2-amino butyric acid, γ-

amino butyric acid, ε-amino hexanoic acid, 6-amino hexanoic acid, 2-amino isobutyric acid, 3-amino propionic acid, norleucine, norvaline, sarcosine, homocitrulline, cysteic acid, τ-butylglycine, τ-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, C-methyl amino acids, N-methyl amino acids, and amino acid analogues in general. Non-natural amino acids also include amino acids having derivatized side groups. Furthermore, any of the amino acids in the protein can be of the D (dextrorotary) form or L (levorotary) form. Allelic variants of a protein sequence are also encompassed within the scope of the invention.

Substitution of amino acids other than those specifically exemplified in a β-amylase or other protein of the present invention as disclosed herein are also contemplated within the scope of the present invention. For example, non-natural amino acids can be substituted for the amino acids of the exemplified protein, so long as the protein retains substantially the same biological activity as the exemplified protein. Amino acids can be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby a protein having an amino acid of one class is replaced with another amino acid of the same class fall within the scope of the subject invention so long as the protein having the substitution still retains substantially the same biological activity as the exemplified protein. Table 1 below provides a listing of examples of amino acids belonging to each class.

Table 1.

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

The subject invention also concerns variants of the polynucleotides of the present invention that encode a protein that produces, or catalyzes the synthesis of, or results in the

production of maltose or a maltose alcohol. Variant sequences include those sequences wherein one or more nucleotides of the sequence have been substituted, deleted, and/or inserted. The nucleotides that can be substituted for natural nucleotides of DNA have a base moiety that can include, but is not limited to, inosine, 5-fluorouracil, 5-bromouracil, hypoxanthine, 1-methylguanine, 5-methylcytosine, and tritylated bases. The sugar moiety of the nucleotide in a sequence can also be modified and includes, but is not limited to, arabinose, xylulose, and hexose. In addition, the adenine, cytosine, guanine, thymine, and uracil bases of the nucleotides can be modified with acetyl, methyl, and/or thio groups. Sequences containing nucleotide substitutions, deletions, and/or insertions can be prepared and tested using standard techniques known in the art.

Polynucleotides and proteins of the subject invention can also be defined in terms of more particular identity and/or similarity ranges with those exemplified herein. The sequence identity will typically be greater than 60%, preferably greater than 75%, more preferably greater than 80%, even more preferably greater than 90%, and can be greater than 95%. The identity and/or similarity of a sequence can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified herein. Unless otherwise specified, as used herein percent sequence identity and/or similarity of two sequences can be determined using the algorithm of Karlin and Altschul (1990), modified as in Karlin and Altschul (1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.* (1990). BLAST searches can be performed with the NBLAST program, score = 100, wordlength = 12, to obtain sequences with the desired percent sequence identity. To obtain gapped alignments for comparison purposes, Gapped BLAST can be used as described in Altschul *et al.* (1997). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) can be used. *See* NCBI/NIH website.

The subject invention also contemplates those polynucleotide molecules having sequences which are sufficiently homologous with the polynucleotide sequences exemplified herein so as to permit hybridization with that sequence under standard stringent conditions and standard methods (Maniatis, T. *et al.*, 1982). As used herein, "stringent" conditions for

hybridization refers to conditions wherein hybridization is typically carried out overnight at 20-25 C below the melting temperature (Tm) of the DNA hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz, G.A. *et al.*, 1983):

Tm=81.5 C+16.6 Log[Na+]+0.41(%G+C)-0.61(% formamide)-600/length of duplex in base pairs.

Washes are typically carried out as follows:

(1) Twice at room temperature for 15 minutes in 1x SSPE, 0.1% SDS (low stringency wash).

(2) Once at Tm-20 C for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

As used herein, the terms "nucleic acid" and "polynucleotide sequence" refer to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, would encompass known analogs of natural nucleotides that can function in a similar manner as naturally-occurring nucleotides. The polynucleotide sequences include both the DNA strand sequence that is transcribed into RNA and the RNA sequence that is translated into protein. The polynucleotide sequences include both full-length sequences as well as shorter sequences derived from the full-length sequences. It is understood that a particular polynucleotide sequence includes the degenerate codons of the native sequence or sequences which may be introduced to provide codon preference in a specific host cell. Allelic variations of the exemplified sequences also come within the scope of the subject invention. The polynucleotide sequences falling within the scope of the subject invention further include sequences which specifically hybridize with the exemplified sequences. The polynucleotide includes both the sense and antisense strands as either individual strands or in the duplex.

The subject invention also concerns plants, plant tissue and plant cells transformed with or bred to contain a polynucleotide that encodes a protein that produces, or catalyzes the synthesis of, or results in the production of maltose or a maltose alcohol. In an exemplified embodiment, the polynucleotide encodes a β-amylase enzyme, or an enzymatically active fragment thereof. Optionally, the β-amylase is one that exhibits reduced inhibition by maltose and/or exhibits increased thermostability. Plants, plant tissue and plant cells expressing a

polynucleotide of the subject invention exhibit increased tolerance or resistance to environmental stresses, including heat stress, cold stress, water stress, and salt stress. In one embodiment, the polynucleotide is overexpressed in the plant cell relative to a plant that has not been transformed or bred to contain a polynucleotide of the invention. Preferably, the polynucleotide is expressed primarily during periods of environmental stress, such as heat or cold. Thus, in one embodiment, a plant comprises a polynucleotide of the invention wherein the coding region of the polynucleotide is operably linked to a promoter that is inducible at a particular temperature range, for example, a temperature that would be cold or a temperature that would be hot to a particular species of plant. In another embodiment, a plant comprises a polynucleotide of the invention wherein the coding region of the polynucleotide is operably linked to a cold temperature inducible promoter, and also comprises a polynucleotide of the invention wherein the coding region of the polynucleotide is operably linked to a heat inducible promoter. In a further embodiment, a polynucleotide of the invention comprises a promoter that is both heat and cold inducible.

Plants within the scope of the present invention include monocotyledonous plants, such as rice, wheat, barley, oats, rye, sorghum, maize, lilies, banana, pineapple, turfgrass, gladiolus, and millet, and dicotyledonous plants, such as cotton, peas, alfalfa, chickpea, chicory, clover, kale, lentil, prairie grass, soybean, tobacco, potato, sweet potato, radish, cabbage, rape, apple trees, coffee, tomato, melon, citrus, beans, roses, sugar beet, squash, peppers, strawberry, carnation, chrysanthemums, impatiens, eucalyptus, and lettuce. In one embodiment, the plant is one that is sensitive to or otherwise adversely impacted by exposure to cold and/or hot temperature. Techniques for transforming plants with a gene are known in the art and include, for example, *Agrobacterium* infection, biolistic methods, *etc.*

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Material and Methods

Plant growth and heat and cold shock treatment.

Arabidopsis thaliana growth conditions: *Arabidopsis thaliana* (var. Colombia) seeds were stratified at 4°C for 3 days and grown in Fafard 2 mix soil (Canadian Sphagnum Peat (55%), Perlite, and Vermiculite) with a 15/9 hr light/dark cycle at 20°C + 2 for 16-18 days. The light intensity was 25-40 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR). Six days before the experiment, light intensity was increased to 90-140 μmol m^{-2} s^{-1} PAR. Plants were exposed the same light intensity 90-140 μmol m^{-2} s^{-1} PAR during heat and cold shock.

Step-up and a step-down experiments: Sixteen-day old Arabidopsis plants were exposed to 25, 30, 35, 40 and 45°C for 1 hr for heat shock and 15, 10, 5, and 0°C for 12 hrs for cold shock and in both experiments, 20°C grown plants were used as a control. Leaf samples were taken 2 hr after the lights were on for RNA extraction for RT-PCR analyses.

Time course experiment: Eighteen-day old Arabidopsis plants were exposed to 40°C for 0, 30, 60, 120 and 240 min for heat shock and to 5°C for 0, 6, 24, 48, 96 and 192 hr for cold shock. Leaf samples were taken 2 hr after the lights were on for RNA extraction for RT-PCR analysis, carbohydrate analysis, western blot, enzyme assay and chlorophyll analysis.

Pea growth conditions: Pea seeds variety Progress #9 (J. W. Jung Seed Company) were soaked in flowing water for about 8 hr before planting. The next day, imbibed seeds were planted on vermiculite and placed in controlled environment at 18°C ±2, 150 μmol m^{-2} s^{-1} PAR with a 12/12 light/dark cycle for 9-10 days.

RNA Extraction.

Heat and cold shocked Arabidospis leaf tissues were flash frozen in liquid N_2 and stored at -80 freezer. Leaf tissues were ground in fine powder in liquid N_2 using pestle and mortar, then extracted using QIAGEN RNeasy Plant Mini Kits (QIAGEN) according to manufacturer's protocol. RLC buffer was used as a lysis buffer in this kit. RNA was quantified using UV spectrophotometry and stored at -80 freezer.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).

Ready To Go RT-PCR Beads (Amersham Pharmacia Biotech) were used for RT-PCR. Each 25 µl RT-PCR included 1 Unit Taq DNA polymerase, 10 mM pH 9 Tris-HCl, 60 mM KCl, 1.5 mM $MgCl_2$, 200 µM each dNTP, Moloney Murine Leukemia virus reverse transcriptase, RNAguard RNase inhibitor (porcine), stabilizers, DNase and RNase-free BSA, 1 µg primer deoxy nucleotide ($pd(N)_6$) for the first strand synthesis, 0.4 µM each gene specific forward and reverse primers (Table 2), 0.1 µM each 18S rRNA forward and reverse primer (internal loading control), 0.3 µM each 3′ terminal dideoxy 18S rRNA forward and reverse primers, and 16 ng total RNA. After addition of total RNA and $pd(N)_6$, each reaction mix was incubated at 42°C for 30 min for the first strand cDNA synthesis. Then gene specific and 18S rRNA primers (as a loading control) were added and PCR amplification was carried out with a Stratagene Robocycler (Stratagene). PCR cycles were:

first cycle: 95°C for 5 min, 95°C for 30 sec, 52°C for 1 min, 72°C for 1 min;

second cycle: 95°C for 30 sec, 52°C for 1 min, 72°C for 1 min which is repeated 25-40 times depending on the gene of interest;

final cycle: 72°C for 7 min.

Then PCR products were kept at 5°C. PCR products were fractionated in a 1% agarose gel. Gels were stained with EtBr and digitally recorded.

Table 2. List of primers used in RT-PCR reactions.

Gene of Interest	Accession #	Primer #	Primers 5′ to 3′	
BMY1	At4g15210	CG363	ACGCCGGAGAATACAATG (SEQ ID NO. 1)	F
		CG364	CAACGGCACAATCTCATG (SEQ ID NO. 2)	R
BMY7	At3g23920	CG305	GACACCCAGTTCAAAA (SEQ ID NO. 3)	F
		CG306	CTCAACTTCTTCCCGACA (SEQ ID NO. 4)	R
BMY8	At4g17090	CG307	GGAACAAGCGGACCTCAT (SEQ ID NO. 5)	F
		CG308	TCTCAGCGATCTTGCCTT (SEQ ID NO. 6)	R
BMY9	At4g00490	CG382	GCTGGCAGGCGTAACACT (SEQ ID NO. 7)	F
		CG383	CGGTTTGAGGAGTTGTAGAAG (SEQ ID NO. 8)	R
IMY	At2g39930	CG351	CGTCTTGAACCACACAGC (SEQ ID NO. 9)	F
		CG352	GCAAAGTCTCCCTCCTCT (SEQ ID NO. 10)	R
AMY	At1g69830	CG345	CCAGGGTAGAGGAAACAA (SEQ ID NO. 11)	F
		CG346	TCGAAGAAGACCGCTGGT (SEQ ID NO. 12)	R
PHOS b	At3g29320	CG315	AAGATGAAGGAAATGAGTG (SEQ ID NO. 13)	F
		CG316	CATCTTTTCTGGTCTCGG (SEQ ID NO. 14)	R
P5CS	At2g39795	CG353	GGACCAAGGGCAAGTAAG (SEQ ID NO. 15)	F
		CG354	AGCCCATCCTCCTCTGTG (SEQ ID NO. 16)	R
SPS	At5g11110	CG321	AATGACAATATCTGAGACTC (SEQ ID NO. 17)	F
		CG322	ACCACATTCTTTAGCCTC (SEQ ID NO. 18)	R
RD29A or Cor78	At5g52310	CG309	CTTTGACTCTGTTCTCGGT (SEQ ID NO. 19)	F
		CG310	GTTGTCAGTTTCTCCGCC (SEQ ID NO. 20)	R
Hsp70	At3g12580	CG258	TCAAGCGGATAAGAGTCACT (SEQ ID NO. 21)	F
		CG259	CTCGTCCGGGTTAATGCT (SEQ ID NO. 22)	R
18S rRNA	At3g41768	CG359	GGAGCGATTTGTCTGGTT (SEQ ID NO. 23)	F
		CG360	TGATGACTCGCGCTTACT (SEQ ID NO. 24)	R
18S rRNA	At3g41768	CG361	GGAGCGATTTGTCTGGTT-3' (SEQ ID NO. 25)	F
3′ dideoxy		CG362	TGATGACTCGCGCTTACT-3' (SEQ ID NO. 26)	R

Chlorophyll Extraction.

Chlorophyll content was determined by the method of Bruinsma (1963). Chlorophyll was extracted from 10 mg freeze-dried tissues using 1 ml of 80% acetone at 4°C overnight in a two ml screw cap micro tube in the dark. Chlorophyll content was quantified spectrophotometrically at 645 and 663 nm.

Carbohydrate Analysis.

Soluble sugar analysis: Eighteen day-old cold and heat stressed Arabidopsis plants were harvested, flash frozen in liquid N_2 and freeze-dried. Lactose 200 μM was added to samples as

an internal standard at the beginning of extraction to normalize the data due to losses during the extraction procedure and due to changes in the detection system of HPLC. Soluble sugars were extracted five times using hot 80% aqueous ethanol from 30 mg (dry-weight) of above ground tissue, including leaves and stems. Ethanol insoluble materials were saved for starch analysis. Ethanol was evaporated at 80°C and the remaining aqueous solution was lyophilized and resuspended in distilled water. To collect soluble neutral sugars, extracts were passed through an Amberlite ion exchange column, which has 1 meq/exchange capacity, at room temperature. The flow-through was freeze-dried. Monosaccharides, which and disaccharides were separated with a NaOH gradient using a Dionex HPLC PA10® column. Monosaccharides, which included fructose and glucose, and disaccharides, which included sucrose, trehalose, maltose and maltitol (sugar alcohol form of maltose), were quantified.

Starch analysis: Starch content was determined by the method of Li *et al.* (1965). After soluble sugar extraction, the EtOH insoluble residue was vacuum-dried. Starch was solubilized in boiling water for 15 min. The supernatant was reacted with iodine-potassium iodide (0.1%) and color density was measured at 620 nm using spectrophotometer. Potato starch was used as standard to estimate starch quantity.

Compatible Solute Assay for Maltose.

SspI (New England Biolabs) is a bacterial restriction enzyme that cuts double stranded DNA. In this assay it cuts pGEX-4T-2 circular plasmid twice and generates 3.77kb and 1.19kb bands in the reaction buffer (50 mM NaCl, 100 mM Tris-HCL, 10 mM $MgCl_2$, 0.025% Triton X-100 pH 7.5 at 25°C). Five units of SspI in reaction buffer in the absence of maltose were exposed to 50°C for 15 min where 50% of the enzyme activity was lost and also exposed to heat temperature in the presence of 14, 100, 200 and 400 mM maltose. Then substrate, 0.5 µg of pGEX-4T-2 circular plasmid (Amersham Biosciences, Piscataway, NJ), was added to the reaction mixture and incubated at 37 °C for 1 hr. The control reaction did not include maltose and was not exposed to heat shock to show the full enzyme activity. To visualize the products, 1% agarose gel was run and 22 µl of total 50 µl reaction was loaded on to gel. The gel was run at 100 V for 30 min and stained with EtBr to visualize the product. The top band was quantified to assess the protection by maltose. Also, the same assay was done using sucrose, trehalose and

glucose to compare the ability of maltose as a compatible solute. A time course experiment was conducted where SspI was treated in the absence and in the presence of 400 mM maltose, glucose, sucrose and trehalose at 50°C for 0, 5, 10, 15, 20 and 25 min.

Glucose-6-phosphate dehydrogenase (G6PDH): Lyophilized G6PDH from *Leuconostoc mesenteroides* was purchased from Worthington Biochemical Company. G6PDH in 50 mM Tris-HCl pH 7.8 was exposed to 48°C for 15 min, which reduced activity by 50%. G6PDH in 50 mM Tris-HCl pH 7.8 was heat shocked in the absence and presence of 14, 100, 200, and 400 mM sugars: maltose, trehalose, glucose and sucrose. The control did not include any sugar and was not given a heat shock. The reaction (1.5 ml) included 2.97 mM $MgCl_2$, 50 mM Tris-HCl pH 7.8, 0.6 mM β-$NADP^+$ (freshly prepared), 10 mM Glucose-6-phosphate , and 595 ng heat shocked G6PDH. Production of NADPH was followed for 3 min at 340 nm using Lambda 3A UV/VIS spectrophotometer (PERKIN-ELMER). A time course experiment with two replications was conducted where G6PDH was treated in the absence and in the presence of 400 mM maltose, glucose, sucrose and trehalose at 48°C for 0, 5, 10, 15, 20 and 25 min.

Alcohol dehydrogenase (ADH): Lyophilized ADH from yeast 300 U/mg was purchased from Calbiochem. ADH in 50 mM potassium phosphate buffer pH 7.6 was exposed to 53.5°C for 15 min, where loss of 50% activity occurs. ADH in 50 mM potassium phosphate buffer pH 7.6 was heat shocked in the absence and presence of 14, 100, 200, and 400 mM sugars: maltose, trehalose, glucose and sucrose. The control did not include any sugar and was not given a heat shock. Reaction volume 1.5 ml included 333 mM EtOH, 50 mM potassium phosphate pH 7.6, 4.15 mM β-NAD^+ (freshly prepared), and 500 ng heat shocked ADH. Production of NADH was followed for 20 s at 340 nm using Lambda 3A UV/VIS spectrophotometer (PERKIN-ELMER). A time course experiment was conducted where ADH was treated in the absence and in the presence of 400 mM maltose, glucose, sucrose and trehalose at 53.5°C for 0, 5, 10, 15, 20 and 25 min.

Pea Chloroplast Isolation.

Pea chloroplast was isolated according to Cline (1993). Aerial portions of 50 g pea plant were chopped into small pieces into 200 ml of ice cold GR-buffer (50 mM HEPES/ adjust pH 7.5 with KOH, 0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 5 mM Na-ascorbate, 1%

BSA) and homogenized using a probe (named PTA 35/2m). The homogenate was filtered through 1 layer of Mira cloth and centrifuged at 2000 x g for 3 min in a swing-out rotor. Pellet was resuspended in GR buffer, overlayed on a Percoll gradient and centrifuged at 2000 x g for 15 min in a swing out rotor. Intact plastids were collected, diluted three times with buffer (50 mM HEPES/KOH pH 8, 0.33 M sorbitol), and pelleted 1500 x g for 5 min. The pellet was resuspended in 25 ml of buffer (50 mM HEPES/KOH pH 8, 0.33 M sorbitol) and chlorophyll content was quantified.

Thylakoid Isolation.

Thylakoids were isolated according to Santarius (1996). The isolated chloroplast pellets were ruptured by resuspending at 1 mg/ml in 10 mM Hepes/KOH, pH 8, 5 mM $MgCl_2$, and incubating 2 min on ice. Then, wash buffer 1:1 (70 mM KCl, 30 mM $NaNO_3$, 20 mM K_2SO_4, 5 mM $MgCl_2$, and 5 mM Hepes/KOH pH 7.5) was added and centrifuged 3300 xg for 8 min to collect thylakoid membranes. The pellet was washed at 1 mg/ml in wash buffer and centrifuged at 3300 x g for 8 min. Thylakoids were resuspended in wash buffer corresponding to 1 mg/ml chlorophyll.

In Vitro Freezing Stress and Heat Shock of Electron Transport Chain.

Heat shock: Aliquots of 0.2 ml thylakoid in wash buffer (70 mM KCl, 30 mM $NaNO_3$, 20 mM K_2SO_4, 5 mM $MgCl_2$, and 5 mM Hepes/KOH pH 7.5) corresponding to 0.5 mg/ml chlorophyll were exposed to heat shock at 40°C for 4 min in the absence and presence of 2.5, 14, 28, 56, and 112 mM maltose, glucose and trehalose. Full activity of the whole electron transport chain was measured after isolation of thylakoid membranes. Five independent experiments with 3 replications each were done.

Freezing stress: Aliquots of 0.2 ml thylakoid in wash buffer (70 mM KCl, 30 mM $NaNO_3$, 20 mM K_2SO_4, 5 mM $MgCl_2$, and 5 mM Hepes/KOH pH 7.5) corresponding to 0.5 mg/ml chlorophyll were exposed to freezing stress at -15°C for 20 hrs in absence and presence of 2.5, 14, 28, 56, and 112 mM maltose, glucose and trehalose. Full activity of the whole electron transport chain was measured after isolation of thylakoid membranes. Four independent experiments with three (3) replications each were done.

Whole electron transport chain assay with DCPIP: This assay was done according to Hipkins and Baker (1986). Assay buffer included 0.1 M sorbitol, 50 mM Hepes-KOH pH 7.6, 5 mM NaCl, 5 mM $MgCl_2$, 0.1 mM DCPIP. The reaction was mixed after addition of thylakoid corresponding to 25 μg/ml chlorophyll and illuminated for 10 sec at 200 μmol photons m^{-2} sec^{-1} light intensity. Activity of electron transport chain was determined using redox dye DCPIP reduction at 595 nm.

All the experiments were done in three replications, unless otherwise specified.

Example 1—Heat and cold shock elicit specific β-amylase gene induction

A step-up and step-down experiment was performed to understand how temperature influences β-amylase expression. Plants were exposed to temperatures from 20° to 40°C for 1 hr in the step-up experiment, and for the step-down experiment, plants were exposed to temperatures from 20° to 0°C for 12 hr (Figure 1). Gene specific transcript levels were evaluated by RT-PCR. Hsp70 was used as a control for heat treatment, and its expression gradually increased as temperature increased (Figure 1). Likewise, Cor78 was used as a control for cold shock treatment and its expression showed slight induction at 10°C and strong induction at 5 and 0°C (Figure 1). BMY8 (Figure 1) showed the greatest expression at 5° and 0°C increasing by 15- and 13-fold, respectively. Expression was more modestly induced at 10°C; approximately 7-fold. BMY8 expression was unchanged under a variety of heat shock temperatures from 20° to 45°C. Conversely, BMY7 expression was markedly increased at 40° and 45°C; 11- and 8-fold, respectively. Expression was not changed under cold shock temperatures down to 0°C. A third predicted chloroplast-localized beta-amylase (BMY9) did not exhibit any temperature-regulated modulation of expression, but instead was expressed at all temperatures from 45° to 0°C.

The major β-amylase in Arabidopsis is a vacuolar form. For comparison, expression of the vacuolar beta-amylase (BMY1) was also tested and its expression was largely unchanged under heat stress, except at 45°C where expression became undetectable. BMY1 expression during cold shock decreased 5 fold at 10° and 5°C, and became undetectable at 0°C.

To determine whether modulation of BMY7 and BMY8 expression is a general stress response for starch degrading enzymes, expression profiles for key enzymes in starch degradation pathways were tested in the step-up and step-down experiments using RT-PCR

(Figure 1). Alpha-amylase (AMY) was repressed 3-fold at 40°C and 5-fold at 45°C; however, its expression was modestly induced 2-fold at 10° and 5°C. Phosphorylase b (Phos b) expression was unchanged under heat stress, repressed 5-fold at 5 and 0°C. Debranching enzyme, isoamylase (IMY) expression was repressed 4-fold at 45°C, but unchanged at all other heat shock temperatures. During cold shock, IMY expression was unchanged except at 0°C where it was repressed 3-fold. Therefore, induction of beta-amylase BMY7 and BMY8 expression under a variety of heat (from 20° to 45°C) and cold (from 20° to 0°C) shock temperatures appears to be a specific response to temperature stress.

Sucrose phosphate synthase (SPS) and delta-1-pyrroline-5-carboxylate synthetase (P5CS) expression profiles were also compared with beta-amylase expression pattern because their transcripts were known to accumulate during cold shock temperatures. SPS expression levels were gradually increased (Figure 1), when the temperature was lowered; 3-, 7-, and 10-fold induction at 10°, 5°, and 0°C, respectively. Under heat shock, SPS expression levels were slightly increased. P5CS expression was induced 2.5- fold at 5° and 0°C, but its expression was unchanged under heat shock, except for a slight decrease at 40° and 45°C (Figure 1).

Example 2—Beta-amylase expression is correlated with maltose accumulation during time course

Based on the previous experiment where time was kept constant and plants were exposed to temperature extremes, β-amylase transcripts were found to be most abundant at 40° and 5 °C under heat and cold stress, respectively. RT-PCR (Figure 2) was performed to show β-amylase transcript levels increase during a time course upon exposure to heat and cold stress. Confirming the previous RT-PCR analysis, under cold shock at 5°C, BMY8 expression was induced as early as 6 hr and peaked at 24 hr, then gradually decreased, but still remained higher than control levels at 192 hr. BMY8 expression during heat shock 40°C was repressed after 60 min. BMY 7 expression peaked at 60 min of exposure to 40°C and then decreased. It did not show any significant change in expression in response to cold shock. Figure 2, shows that after 6 hr at 5°C, BMY7 expression seemed to be induced, however it was seen only in one replication of the three. BMY9 and BMY1 failed to show significant changes in expression under heat shock condition for about 2 hours; after that, BMY9 expression slightly decreased and BMY1

expression was induced for 7 fold. At 5°C, BMY9 and BMY1 expression were unaffected. As a control for heat treatment, Hsp70 was used. Its expression peaked at 30-60 min upon exposure to 40°C. Under cold shock conditions, Hsp70 also exhibited a slight induction until 24 hr, after which its expression became undetectable. Hsp70 is known to be induced modestly in response to cold shock. As a cold shock treatment control, Cor 78 was used. It showed a very similar expression profile to BMY8 under cold shock 5°C, but no significant changes under heat shock conditions.

Leaf tissue maltose content was characterized to determine whether maltose accumulation paralleled β-amylase expression. Soluble sugar analysis (Figure 3) revealed that maltose content doubled from 0.06 to 0.14 μmol mg^{-1} Chl within 30 min exposure to 40°C, remained constant until 60 min, and then gradually decreased back to control levels at 4 hr. The maltose accumulation profile (Figure 3) was very similar to that of sucrose. During cold shock at 5°C, the maltose content increase was much more dramatic from 0.04 (control) to 0.60 μmol in the first 6 hr then continued to accumulate to 0.80 μmol mg^{-1} Chl by 48 hr. Afterward it gradually decreased to 0.11 μmol mg^{-1} Chl by 192 hr. The maltose accumulation profile (Figure 3) was similar to that of sucrose, glucose and fructose. When soluble sugar content began decreasing, trehalose content started increasing about 96 hr after cold shock. In contrast, trehalose content slightly decreased after 90 min of exposure to 40°C.

Intact chloroplast were isolated from 10 day-old pea leaves exposed to 40°C for 30 min or to 5°C for 24 hr to show whether maltose actually accumulates in the chloroplast under temperature stress. Maltose content increased in pea chloroplasts by about 2.5 fold; but overall, the maltose content was about one order of magnitude less than that of Arabidopsis.

Starch content was also determined in order to better understand its relationship with maltose accumulation. Starch content increased gradually and was correlated with accumulation of maltose at about 48 hr during cold shock. Accumulation of starch became pronounced after 96 hr of cold shock; however, this was not followed by maltose accumulation. Conversely, during heat shock at 40°C, starch content decreased over time from 0.15 to 0.04 mg mg^{-1} Chl.

Example 3—Maltose has compatible solute properties

Compatible solutes can stabilize proteins and membranes and contribute to cell osmotic potential during stress. The compatible solute potential of maltose was tested for three different enzymes namely; SspI, Glucose-6-phosphate dehydrogenase (G6PDH) and alcohol dehydrogenase (ADH). The restriction enzyme, SspI (Figure 4A), cuts the substrate plasmid (pGEX-4T-2; 4.97 kb) twice leading to 1 and 4 kb plasmid fragments, when the enzyme is fully functional. If the enzyme is compromised by high temperature, it does not completely digest the plasmid, but produces a single cut plasmid fragment of 5 kb (top band) band and double cut fragments of 1 and 4 kb (Figure 4A); as a result, 3 bands are seen instead of 2 bands. When the intensity of the 5 kb top band and the 4 kb band was equal, the enzyme was considered to have lost 50 % activity. Ssp1 was exposed to 50°C for 15 min (Figure 4A) in the absence of maltose (0 mM as a control) and in the presence of (14, 100, 200, and 400 mM) maltose. The 5 kb top band, which represented the loss of activity, was quantified to determine relative protection. When the enzyme was fully active, the top band was not visible (Figure 4A, lane C) and the intensity of the top band increased with increased loss of enzyme activity (lane 2; 0 mM sugars). At a physiological concentration, maltose (14 mM) was able to partially protect SspI function. This protection increased with the increasing maltose concentration (Figure 4A). At 200 and 400 mM, the intensity of the top band was much less compared to 0 mM maltose.

Besides maltose, identical concentrations of sucrose, trehalose and glucose were also tested to compare performance of maltose as a compatible solute (Figure 4A). Maltose performed as well as trehalose at physiological concentration. It was the second best after trehalose and superior to glucose and sucrose. At 200 mM, trehalose showed 100 % protection, while the other sugars, including maltrose, showed around 80-90% protection. At 400 mM, glucose, trehalose, and sucrose showed 100% protection, while maltose showed around 85-90 % protection.

In addition, protection of SspI activity (Figure 7A) in the presence of the soluble sugars maltose, trehalose, glucose and sucrose (400 mM) can be maintained at least for 25 min at 50°C, while in the absence of sugars the enzyme lost almost 90% activity over time. Trehalose, glucose and sucrose show 100 % protection, while maltose exhibited about 90% protection.

To test whether this protection is general, compatible solute assays were performed using G6PDH, which uses D-glucose-6-phosphate and $NAD(P)^+$ as substrates to produce D-glucono-δ-lactone-P and NAD(P)H (Figure 4B). Maltose performed as well as the other compatible solute sugars even at physiological concentration. The same performance was seen (Figure 7B), when the time was extended to 25 min in the presence of 400 mM of the above sugars.

ADH utilizes RCH_2OH and NAD^+ as substrates to produce RCHO, NADH and H^+. ADH was heat stressed (Figure 4C) in the absence and presence of (14, 100, 200, and 400 mM) maltose, glucose, trehalose, and sucrose. Similar to SspI and G6PDH compatible solute assays, maltose showed a similar level of protection with ADH. The same was true when the time was extended to 25 min at 53.5°C (Figure 7C).

Example 4—Maltose can function as a chloroplast stromal compatible solute *in vitro*

Maltose, trehalose and glucose were tested *in vitro* for compatible solute properties using thylakoid membranes for the functionality of electron transport chain against heat denaturation (Figure 5) and freezing stress (Figure 6). Pea thylakoids were exposed to 40°C for 4 min (Figure 5), where 30% of electron transport chain activity remained in the absence of compatible solute sugars. Electron transport chain activity was followed by reduction of a redox dye 2,6-dichlorophenolindophenol (DCPIP), which accepts electrons from Q_B of PSII and FeS_A of PSI (Häder and Tevini, 1987). At physiological concentrations of 2.5 and 14 mM maltose, photosynthetic electron transport chain activity was protected 3 and 5%, respectively, during heat shock. When the concentration of maltose increased, activity was preserved up to 45% at 112 mM, which is very comparable to the known compatible solute trehalose 54% at the same concentration. Glucose showed 30% protection at 112 mM.

Pea thylakoids were frozen (Figure 6) at -15°C for 20 hr, which caused a 60% reduction of electron transport chain activity. Similar to heat shock, protection by maltose was seen at very small maltose concentrations of 2.5 and 14 mM, which preserved 2 and 24% of activity, respectively. This protection increased gradually to 93% with the increasing concentration of maltose and was very similar to that of trehalose. Glucose at 112 mM gave 47% protection, which was much less than that of maltose or trehalose.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

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